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Yeast Two-Hybrid System and its Advances in Protein Interaction Study

Sangh Chandramohan[1]* and Veena Gupta[2]

[1]Division of Plant Genetic Resources, Indian Agricultural Research Institute, Pusa campus, New Delhi - 110 012, India.
[2]Division of Germplasm conservation, National Bureau of Plant Genetic Resources, Pusa campus, New Delhi - 110 012, India.

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With the advent of new sequencing technologies, genome sequencing efforts are speeding up the discovery process at a faster rate. With this explosion of sequence information, there is a need to understand how genes work in concert in order to fulfill the cells functions. The yeast two-hybrid system used to identify protein-protein interactions is one of the most powerful and versatile methods for characterizing a protein's function. Yeast two hybrid systems is one of the basic technologies used in protein interaction studies for both academic researchers and those in biotechnology and pharmaceutical companies. Most of interaction studies reported in literature gives an insight about molecular mechanism underlying the biological processes. Several advancements made over the original Yeast two hybrid systems, which will help in understanding and revealing many facts in the complex field of protein interactions. In this review we discussed about the yeast two hybrid system and its advances in protein interaction.

Key words: Yeast two hybrid, BiFC, FRET, BRET, LuCI.

The phenomenon of protein–protein interaction occurs in all cellular processes viz from DNA replication to signal transduction, cell-cycle control and intermediary metabolism. In most cases, the interactions among proteins are dynamic and the knowledge of assembly and disassembly of them over time in response to complex signals helps one to perceive the processes that make up life. The detailed characterization of macromolecular interactions will also help one to identify the defective pathways of tissues in pathological state and the infective mechanisms of pathogens. The yeast two-hybrid system has become one of the most popular and powerful tools to study protein–protein interactions. With the advent of proteomics, the two-hybrid system has found a niche in interactome mapping.

The availability of high-throughput methods for proteomics research will dramatically increase our knowledge of protein interaction networks. The two most frequently used methods are yeast two-hybrid (Y2H) screening, a well established genetic *in vivo* approach, and affinity purification of complexes[1]. Understanding protein function is key to understanding how complex

* To whom all correspondence should be addressed.
E-mail: chandramohan8207@gmail.com

biological systems operate in normal physiological situations and for understanding how these systems are dysregulated in pathological conditions. Identifying the function of a protein can be very difficult to achieve, particularly for novel proteins identified, for instance, by genome sequencing technologies or by immunological or proteomic approaches. Many clues for functionality can be provided by a careful analysis of the expression patterns of the protein in a variety of circumstances[2].There are number of considerations that must taken into account while using yeast two hybrid system viz. false positives, post translational modification etc.

There are various methods to study protein–protein interactions. Biochemical methods includes Co-immunoprecipitation, Bimolecular Fluorescence Complementation (BiFC) Affinity electrophoresis, Label transfer, Yeast two-hybrid, Photo-reactive amino acid analogs, Tandem affinity purification (TAP), Chemical cross linking, Quantitative immune-precipitation combined with knock-down (QUICK). Biophysical and theoretical methods are Dual polarisation interferometry (DPI), Static light scattering (SLS), Surface plasmon resonance, Fluorescence correlation spectroscopy, Fluorescence resonance energy transfer (FRET), Theoretical modeling, Molecular dynamics (MD), Protein-protein docking. Each method has their own set of advantages and disadvantages, depending on type of protein under study.

These technologies assist in revealing the functions of newly identified proteins, characterization of different signal transduction and metabolic pathways, identification of new drug targets and generation of insight in the complexity of a biological process, determination of protein function, verifying an interaction between two known proteins or protein domains for which there is a prior reason to expect an interaction (testing two-hybrid interactions), screening a library for novel proteins that specifically interact with a known bait, identifying mutations that affect complex formation between two proteins known to interact specifically.

Yeast Two-Hybrid system

Pioneered by Fields and Song in 1989, the technique was originally designed to detect protein–protein interactions using the GAL4 transcriptional activator of the yeast *Saccharomyces cerevisiae*. The GAL4 protein activated transcription of a protein involved in galactose utilization which formed the basis of selection[3].

The yeast two-hybrid system is based on a hallmark feature of eukaryotic transcription factors: these proteins consist of a DNA binding domain (DBD) which recognizes and binds to a defined promoter sequence upstream of a gene and an activation domain (AD) which interacts with the RNA polymerase II complex. The DBD positions the transcription factor upstream of its target gene and the AD then recruits the RNA polymerase II complex to the start of the gene, thereby activating its transcription. In the first yeast two-hybrid systems, fragments of the yeast gene *GAL4* were cloned to make the DBD and AD. The Gal4p is a transcription factor of the yeast galactose metabolism pathway, initiating over 1,000 fold expression of both *GAL1* and *GAL10* (4). Therefore, cloning the promoters for either *GAL1* or *GAL10* upstream of a suitable gene creates a dynamic reporter for the interaction in question. The galactose pathway is still the biological basis for most yeast hybrid systems, including the conditional expression of proteins DBD-bait, AD-prey in many experiments. However, the Gal4 DBD and AD have for many investigators been replaced by DBDs and ADs from other organisms, commonly the LexA DBD and B42 AD from *E. Coli* (5). A LexA-based yeast two-hybrid system is sometimes referred to as a yeast interaction trap. These changes were made in part to prevent the system from disturbing normal cellular function due to overexpression of *GAL4* domains[6].

When the DBD and the AD are expressed as separate polypeptides, the function of the transcription factor is lost: the DBD still binds to its cognate promoter sequence but is unable to activate transcription. The AD can still interact with the RNA polymerase II complex, but since it is not located near the gene anymore, no transcriptional activation is taking place.

The yeast two-hybrid system takes advantage of the modular setup of a transcription factor. In the first step, a cDNA encoding your protein of interest is cloned into a bait vector, creating a fusion of the DBD and your protein of interest. This fusion protein (termed the bait) translocates to the nucleus of the yeast cell

and binds to the promoter located upstream of a reporter gene. As this bait lacks an activation domain, no activation of the downstream reporter genes takes place.

A second cDNA encoding an interacting protein (or a library of cDNAs encoding an entire collection of different potential interactors) is cloned into a prey vector, creating a fusion of the AD and the interacting protein. The fusion protein (termed the prey) has the potential to activate yeast genes due to the presence of the AD but is unable to so since it is not located near a reporter gene.

If bait and prey interact, the AD is recruited to a reporter gene, creating a hybrid transcription factor. The downstream reporter gene, for example an auxotrophic marker such as HIS3 or ADE2 or a colour marker like lacZ, is transcribed, resulting in histidine or adenine prototrophy (cells expressing HIS3 or ADE2 are able to grow on selective medium lacking the amino acid histidine or the metabolite adenine, respectively) or blue coloration of the yeast cells. Thus, the interaction of two proteins is measured by the reconstitution of a hybrid transcription factor and the consequent activation of a set of specific reporter genes. The power of the yeast two-hybrid system lies in this combined approach since growth selection enables sampling of highly complex cDNA libraries encoding millions of potential binding partners: only those clones which encode an interacting protein survive growth selection are analysed further using the convenient colour assay. The advantages and limitation has been provided in table 1.

cDNA libraries are essential tools for developing expressed sequence tag (EST) databases and exploring an organism's transcriptome. One method is creation of high quality Arabidopsis cDNA libraries and the subsequent transfer of those libraries to yeast two-hybrid (Y2H) vectors[7]). Most signiûcantly, the two-hybrid system can be used to screen libraries of activation domain hybrids to identify proteins that bind to a protein of interest. These screens result in the immediate availability of the cloned gene for any new protein identiûed. In addition, since multiple clones that encode overlapping regions of protein are often identiûed, the minimal domain for interaction may be readily apparent from the initial screen[8-9].

Y2H screens require high quality libraries and are one of the most effective methods of identifying novel protein-protein interactions[10,1,11-12]. Cao[13] *et al*. 2011, reported the creation of two high quality and a publicly available Gateway™ cDNA entry libraries and their derived Y2H libraries for *Brachypodium*. Feng *et al* 2010 [14] developed yeast one-hybrid system to screen cDNA libraries for clones encoding methylated DNA-binding proteins. An HD-Zip IV gene from wheat, *TaGL9*, was isolated using a Y1H screen of a cDNA library prepared from developing wheat grain[15]. Screening of an Arabidopsis cDNA library by yeast-2-hybrid (Y2H) using the J-like domain of CJD1 as bait identified a plastidial inner envelope protein (Accumulation and Replication of Chloroplasts 6, ARC6) as the primary interacting partner[16].

Brown *et al*. 2011 [17] developed two methods to create gene fragments – random fragmentation by partial DNAse I digestion and generation of densely overlapping fragments by PCR. Both approaches revealed a putative interaction between PfMyb2 (PF10 0327) and PFC0365w. The combination of improved yeast two-hybrid screening approaches and convenient systems to validate interactions enhances the utility of yeast two-hybrid assays for *P. Falciparum*.

A derivative of yeast two hybrid is used to study protein-small molecule interactions, termed the yeast three-hybrid system[18], requires only the addition of a small-molecule to mediate bait and prey "dimerization". This compound is thus termed a chemical inducer of dimerization (CID). The CID serves as a third hybrid: a chimera of two moieties, one with affinity to the bait and the other with affinity to the prey . For target screening, this hybrid consists of the drug tagged by an established high affinity ligand for the bait or prey (by convention usually the bait). This high affinity interaction creates a platform, or anchor moiety, for screening cloned proteins against the tethered query compound. An example of a validated anchor moiety is the protein-ligand duo dihydrofolate reductase and methotrexate (DHFR-Mtx) [19], due in part to its picomolar affinity. A system using DHFR-Mtx has been used to successfully screen the mammalian proteome for targets of kinase inhibitors[20]. The timeline and evolution of Y2H is given in table 2.

False positives and false negatives are the two major issues in interaction study. False positives are the candidate proteins which are identified as interacting but which do not truly interact or are biologically irrelevant. It can be rectified by isolating prey and retest with control baits (prey without bait, prey with DBD alone, prey with unrelated DBD hybrid, prey with nonfunctional mutant bait), providing four phenotypes for assessing true interactors, using low-copy-number (*ARS/CEN)* vectors that reduce expression levels and toxicity. Where as in case of false negatives candidate proteins which truly interact will be reported as non interacting proteins. Within the yeast-two hybrid system itself, spurious activation of reporter genes can occur for a number of reasons, leading to false results. For instance, some proteins may themselves bind to DNA or may transcriptionally activate the reporter genes, so that apparent positives are obtained when there is in fact no interaction present. These problems can be reduced by analysing more than one reporter gene and by swapping the two domains in the two proteins. Similarly, the proteins may adopt a different tertiary structure when expressed as fusions with the transcription factor domains, which could potentially inhibit the binding of true protein partners. Also, some proteins may be toxic when expressed as fusions in yeast, thereby inhibiting growth when expressed at high levels; this can be circumvented to an extent by the use of inducible expression plasmids. More important still are situations in which these factors lead to spurious binding of proteins that do not normally bind in *vivo*, thereby leading to false positives. This might be because of the different environments in yeast and mammalian cells, and can be investigated by performing a two-hybrid assay in the context of mammalian cells to confirm that the interactions can indeed occur in mammalian cells. However, since the mammalian two-hybrid assay is based on the same approach, it is subject to the same artefacts. Finally, a major problem with yeast two-hybrid assays is that the two proteins have to be directed to the nucleus for the assay. Thus, proteins that do not normally interact because they are present in different cellular compartments are necessarily brought into proximity in the two-hybrid assay and allowed to interact. Important reasons are given in table 3.

Split-ubiquitin system for membrane protein interactions

The split-ubiquitin membrane yeast two-hybrid (MbYTH) assay utilizes complementation between separable domains of ubiquitin to study membrane protein interactions. Ubiquitin is a small, highly conserved protein, which when covalently attached to a target protein marks that protein for degradation by the 26S proteosome. Whereas the target protein is degraded by the 26S proteosome, the ubiquitin motifs are saved from degradation by the ubiquitin-specific proteases (UBPs), which cleave the ubiquitin from the target protein and hence recycle the ubiquitin back to the cytoplasm (34). The highly specific cleavage of ubiquitin from the target protein is presumably dependent upon the folded structure of ubiquitin. If the wildtype N-terminal fragment of ubiquitin (NubI) (amino acids 1–34, with I representing the isoleucine at position 13) and the C-terminal fragment of ubiquitin (Cub) (amino acids 35–76) are expressed within yeast, the NubI and Cub portions will spontaneously associate and be recognized by the UBPs . Replacing the wild-type isoleucine residue at position 13 of NubI with that of glycine (NubG) decreases the affinity between NubG and Cub compared to that of NubI and Cub. However, if the NubG and Cub moieties are fused to interacting proteins X and Y, the interacting proteins force NubG and Cub into close proximity. This results in partial re-association of ubiquitin, which is recognized by the UBPs[22]. If a suitable reporter is fused to the C-terminus of Cub, association of the Cub-reporter with NubG, followed by recognition and cleavage by the UBPs, will cause reporter release from Cub, leading to its activation.

The reporter format for the MbYTH system is transcriptional activation readout. In the system, a hybrid transcription factor (TF) composed of the bacterial LexA protein and the Herpes simplex VP16 transactivator domain is fused to the Cub domain of ubiquitin (Cub-TF). To detect protein interactions via the MbYTH system, the protein of interest fused to Cub-TF must be membrane associated to prevent the TF domain from entering the nucleus. Upon reconstitution of Cub-TF with NubG via interacting intermediates, the UBPs cleave the TF from Cub, which releases the TF from the membrane and allows its entry into the nucleus where it activates reporter gene

expression. If proteins fused to NubG and Cub-TF fail to bring together the NubG and Cub domains, release of the TF from Cub via the UBPs will not occur and the TF will remain at the membrane unable to activate gene transcription[35].

Bimolecular Fluorescence Complementation

The most widely used approach for the visualization of protein interactions in living cells is fluorescence resonance energy transfer (FRET) between spectral variants of the green fluorescence

Table 1. Advantages And Limitations of The Yeast Two-Hybrid System

Advantages	Limitations
Cheap and simple	Interactions are assayed in the yeast nucleus rather than the correct cellular compartment
In vivo protein interaction analysis	Membrane-bound proteins and transcription are often not suitable (since Y2H forces proteins into the nucleoplasm and relies on transcriptional activation as a read-out).
Capable of detecting weak or even transient interactions	False positives and false negatives can occur
Can be used to identify novel interactors by cDNA library screening	Some of the expressed proteins are toxic to yeast
Several variations allows multiple applications and can be scaled up through automation for genomic-scale protein	Yeast cell environment may not fully mimic mammalian cells

Table 2. Y2H timeline and its evolution

Year	Y2H method	References
1989	Classic Y2H system	Fields, S; Song, O. (3)
1994	SOS recruitment system (SRS)Split-ubiquitin system	Aronheim *et al (21)*Johnsson *et al (22)*
1998	Membrane splitubiquitin systemRas recruitment system	Stagljar *et al (23)*Broder *et al (24)*
1999	Dual bait system	Serebriiskii *et al (25)*
2000	G-protein fusion system	Ehrhard *et al (26)*
2001	RNA polymerase III based two-hybrid	Petrascheck *et al (27)*
	Repressed transactivator system (RTA)	Hirst *et al (28)*
	Reverse Ras recruitment system (rRRS)	Hubsman *et al (29)*
2003	SCINEX-P system	Urech *et al (30)*
2004	Split-Trp system	Tafelmeyer *et al (31)*
2007	Cytosolic split-ubiquitin system (cytoY2H)	Mockli *et al (32)*
2011	Membrane-SPINE	Muller *et al (33)*

Table 3. Reasons for false positives and false negatives

False positives	False negatives
Auto-activation	Protein not represented in library
Proteins containing regions with surfaces having low affinities for many différent proteins	Poorly expressed proteins
Proteins that normally interact with a large number of proteins (e.g., heat shock proteins)	Misfolded bait or prey
Proteins containing regions functioning as activation domains	Mislocalized bait or prey
Proteins having low or nonspecific affinities for the promoter regions that drive the expression of reporter genes	Lack of post-translational modifications and toxic hybrid proteins

protein (GFP) fused to the associating proteins[36]. However, to enable observation and quantification of small alterations in fluorescence emission, the GFP fluorophores have to join in close spatial proximity and the fusion proteins generally have to be expressed in high levels. Furthermore, verification, whether changes in fluorescence emission are caused by energy transfer, requires complicated irreversible photo bleaching or fluorescence lifetime imaging techniques[37]. However, the instrumental equipment necessary for these techniques is not widely available and FRET requires intensive methodical training. For these reasons reports about FRET-based protein–protein interaction investigations in living cells have remained rare especially in plant science[38-41].

Alternatively, protein interactions can also be investigated in vivo if the protein complex formation can be visualized by the restoration of a detectable activity. In this regard, the principle of intragenic complementation of the lacZ locus from Escherichia coli was adapted to detect protein interactions[42-43]. In this experimental system the detection of protein–protein interactions by restoration of b-galactosidase activity was enabled by using b-galactosidase fragments, which could associate only when fused to interacting proteins. Similarly, fragments of the dihydrofolate reductase have been used in protein interaction studies based on complementation of protein function[44]. However, these techniques require the application of extrinsic fluorophores to visualize the complex formation. An alternative experimental approach for the visualization of protein interactions is based on the formation of a fluorescent complex by fragments of the enhanced yellow fluorescent protein (YFP) when brought together by the interaction of two associating partners fused to these fragments.

Recently, a proof-of-concept for an approach for the investigation of protein interactions in living mammalian cells and designated this technique as bimolecular fluorescence complementation (BiFC). The unique characteristic of the BiFC approach is that the bright intrinsic fluorescence of the bimolecular complex allows direct visualization of the complex formation in living mammalian cells. Moreover, by analyzing the interactions between members of the basic leucine zipper (bZIP) and Rel transcription factor families, the BiFC approach provided direct evidence of the intracellular locations where the protein association occurs[45]. The application of the BiFC approach has recently been extended to the investigation of the interaction pattern and intracellular localization of G-protein complexes in mammalian cells and *Dictyostelium discoideum*[46] and to the visualization of 1-aminocyclopropane-1-carboxylase synthase heterodimer formation in *E. coli*[4]). Furthermore, by introducing a large number of different GFP variants the technique was extended to multicolor BiFC, which allows the direct visualization of multiple protein interactions within the same cell[48-49].

Luciferase Complementation Imaging Assay

The development of reporter-based in vivo protein-protein interaction assays, such as fluorescence resonance energy transfer[50-52], the related technology bioluminescence resonance energy transfer[53-54], and bimolecular fluorescence complementation[45] assays, has significantly advanced the measurement of protein-protein interactions in vivo. These assays are instrumental for a number of important discoveries in mammalian studies. The application of FRETand BRET in plant biology, however, has encountered significant difficulties despite sporadic successes. Both assays require sophisticated microscopy and computation. BiFC is relatively simple compared to FRET and BRET and has been used in a number of plant protein-protein interaction studies[55-58]. FRET and BiFC are technically challenging when a large number of protein pairs are to be tested. Furthermore, the application of FRET and BiFC assays in plants is complicated by the autofluorescence generated by cell wall, chloroplast, and other cell structures. Finally, photobleaching and phototoxicity caused by the external light source for excitation of fluorescence also restrict the application of the reporter-based assays in plants[59].

Alternative reporter-based methods for protein-protein interactions have been developed using protein fragment complementation coupled with enzymatic assays. For example, expression of b-galactosidase fragments fused to interacting proteins reconstitutes the enzymatic activity in *Escherichia coli*[42]. Similarly, 1-b-lactamase has been used to detect protein-protein interactions in mammalian cells[60]. Protein fragment

complementation based on the reconstitution of murine dihydrofolate reductase[61] was used to detectNPR1-TGA2 interaction in plants[54]. These assays typically require the addition of fluorescence-generating substrates and thus also suffer from the pitfalls of FRET and BiFC. Recently, an improved firefly luciferase complementation imaging (LCI) assay was developed for protein-protein interactions in animals[62].

The firefly luciferase (LUC) enzyme is divided into the N- and C-terminal halves that do not spontaneously reassemble and function. LUC activity occurs only when the two fused proteins interact, resulting in reconstituted LUC enzyme, which can be detected by luminometer or a low-light imaging device. The assay measures dynamic changes in protein-protein interactions and can be used for both cell culture and whole animals. Because the luminescence was measured in the dark and is not affected by autofluorescence, LCI is particularly attractive for plant studies. Avery recent report successfully used *Renilla reniforms* LUC complementation assay to detect interactions of two pairs of plant proteins in protoplasts[63]. The utility of the firefly LCI in plant protein-protein interaction studies remains to be tested.

CONCLUSION

Once the interaction has been identified and validated, its function in the biological system should be established. These experiments can be carried out for defined interactions of a small number of proteins, but again it would be quite difficult to transfer them to the large interaction network generated by global screens. It also should be emphasized that recent studies of protein–protein interactions, in particular, those involved in signal transduction, uncovered a number of protein-binding domains or motifs, which are evolutionarily conserved and used in various signaling pathways. Despite the need for comprehensive studies on protein–protein interactions, less efforts for a genome-wide scale screening has been made. So far, interactome approaches concentrate on a characterization of the nodes in the interaction network, which may be the major determinants of a phenotype. Combination of a panel of complementary methods is generally able to unveil the physiological significance of an interaction identified in a targeted approach. Hence there is a need to develop suitable technologies for studying the large interaction network.

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